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Characterization of *Diplodia seriata* and *Neofusicoccum parvum* Associated with Grapevine Decline in Iran

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ABSTRACT

During 2004-2007, a field survey was conducted in different vineyards to determine the fungal pathogens associated with grapevine decline in different provinces of Iran including Fars, Hamedan, Isfahan, and Kohgiluyeh and Boirahmad. Twenty-seven isolates of *Botryosphaeriaceae* were recovered from vines showing decline and dieback symptoms only in Fars Province. Based on morphological and cultural characters along with molecular analysis [partial sequences of the nuclear ribosomal internal transcribed spacer (ITS), beta-tubulin (BT), and elongation factor 1-α (EF)], two species of *Botryosphaeriaceae*, namely, *Diplodia seriata* and *Neofusicoccum parvum* were identified. Both species were pathogenic on rooted cuttings of cv. Cabernet Sauvignon and caused the dieback and drying-out of leaves as well as extended lesions on the shoots 4 months after inoculation. Results showed that *N. parvum* isolates were the most virulent and produced significantly longer lesions than those caused by *D. seriata*. Both species were reisolated from the margin of the lesions completing Koch's postulates.

Keywords: *Botryosphaeriaceae*, Internal transcribed spacers, Pathogenicity, Translation elongation 1-α gene, *Vitis vinifera*.

INTRODUCTION

Members of the family *Botryosphaeriaceae* (*Botryosphaeriales*, *Dothideomycetes*, *Ascomycota*) can be parasitic, saprophytic, or endophytic on different monocotyledonous, dicotyledonous, and gymnosperm plant species and are widely distributed geographically (Barr, 1987; Denman *et al*., 2000; Phillips, 2002). The taxonomy and identification of *Botryosphaeriaceae* spp. were based on the anamorph characteristics for many years (Denman *et al*., 2000; Phillips, 2002), since their teleomorphs are rarely formed in nature or in culture (Shoemaker, 1964). According to Denman *et al.* (2000), at least 18 anamorph genera have been associated with the family *Botryosphaeriaceae*, including *Diplodia* Fr., *Fusicoccum* Corda, *Lasiodiplodia* Ellis and Everh., *Dothiorella* Sacc., and *Sphaeropsis* Sacc. However, their identification has become very difficult because of their overlapping characteristics (Slippers *et al*., 2004a, 2004b). For this reason, molecular and morphological characters have been later used to resolve the identification and taxonomic problems of members in the *Botryosphaeriaceae* (Denman *et al*., 2000; Smith and Stanosz, 2001; Zhou and Stanosz,

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2001; Slippers *et al*., 2004a; Crous *et al*., 2006; Phillips *et al*., 2008).

The disease caused by *Botryosphaeriaceae* on grapevines is named black dead arm (BDA) and was first described by Lehoczky (1974), who associated this disease with *B. stevensii* Shoemaker. Several other species of *Botryosphaeriaceae* were later isolated and identified from BDA-affected vines such as *B. dothidea*, *D. seriata,* and *L. theobromae* (Larignon *et al*., 2001; van Niekerk *et al*., 2006). Currently, different anamorphic genera of *Botryosphaeriaceae* include the species of *Fusicoccum*, *Neofusicoccum*, *Diplodia*, *Lasiodiplodia*, and *Dothiorella* occurring on grapevines (Denman *et al*., 2000; Phillips *et al*., 2005; Crous *et al*., 2006). Although the pathogenicity of *Botryosphaeriaceae* members on grapevines has not yet been fully clarified, different symptoms associated with the species have been reported on grapevine worldwide. The common leaf and vascular symptoms associated with *Botryosphaeriaceae* in affected grapevines include wood necrosis, vascular streaking, perennial cankers, trunk dieback, mild chlorosis, bud mortality and wilting of leaves (Phillips, 1998; Larignon and Dubos, 2001; Phillips, 2002; van Niekerk *et al*., 2004). Other symptoms often associated with *Botryosphaeriaceae* species are the formation of a dark stripe on the wood surface just below the bark of affected grapevines (Larignon and Dubos, 2001) and wedge-shaped necrosis (Mugnai *et al*., 1999). *Botryosphaeriaceae* species could have a large economic impact on the grapevine as evidenced by the fact that *D. seriata* alone have been reported as being responsible for losses of up to 20% in the Bordeaux wine region of France (Larignon *et al*., 2001).

In Iran, *D. seriata* has been reported on *Malus pumila* (Viennot-Bourgin *et al*., 1970) and also, as *Sphaeropsis malorum* (Berk.) Berk, from pine trees in Golestan Province, East Gorgan forests, (Nasrollah-Nejad *et al*., 1998). Two new species, *Barriopsis iraniana* Abdollahzadeh, Zare and A.J.L. Phillips (from *Citrus*, *Mangifera* and *Olea*) and *Phaeobotryon cupressi* Abdollahzadeh, Zare and A. J. L. Phillips (from *Cupressus semperivirens*) were also reported from Iran based on morphological and molecular studies (Abdollahzadeh *et al*., 2009). Six species of *Lasiodiplodia* were recently isolated and reported from a range of woody hosts in Iran, namely, *L. pseudotheobromae* A. J. L. Phillips, A. Alves and Crous., (on *Citrus* sp.) and *L. theobromae* (on *Mangifera indica*), and the previously unknown species *L. citricola* Abdollahzadeh, Javadi and A. J. L. Phillips, (on twigs of *Citrus* sp., from Chaboksar, Gilan Province), *L. gilanensis* Abdollahzadeh, Javadi and A. J. L. Phillips (on twigs of an unknown woody plant, from Rahimabad-Garmabdost, Gilan Province), *L. hormozganensis* Abdollahzadeh, Zare and A. J. L. Phillips (on twigs of *Olea*, from Rudan-Kheirabad, Hormozgan Province) and *L. iraniensis* Abdollahzadeh, Zare and A. J. L. Phillips, (on *Mangifera indica*, *Eucalyptus* sp., *Citrus* sp., *Salvadora persica*, *Juglans* sp. and *Terminalia catapa*, from Hormozgan and Golestan Provinces, Southern and Northern Iran) (Abdollahzadeh *et al*., 2010).

Recently, a relatively high occurrence of grapevine decline disease has been observed in different vineyards of Iran (Mohammadi and Banihashemi, 2007; Mohammadi *et al*., 2009; Mohammadi and Banihashemi, 2012; Mohammadi, 2011; Mohammadi, 2012), but to date, there is no information available about the occurrence of black dead arm disease and the associated *Botryosphaeriaceae* species in vineyards in this country. The objective of the present study was to investigate the identity based on morphological and molecular characteristics and pathogenicity of *Botryosphaeriaceae* species associated with grapevine trunk disease in Iran.

MATERIALS AND METHODS

Survey and Sample Collection

During 2004-2007, 41 own rooted grapevine vineyards (4 to 35 years old), located in different provinces of Iran, were surveyed to study the fungi associated with trunk diseases. The number of vineyards investigated in each

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province was: 21 for Fars, 5 for Hamedan, 9 for Isfahan, and 6 for Kohgiluyeh and Boirahmad Provinces. Approximately, 8 plants (cvs. 'Askari', 'Rishbaba' and 'Black') showing yellowing and reduced growth of canes and shoots and different symptoms in wood, including brown wood, necrosis, and brown and black streaking, were sampled from each vineyard. The superficial bark tissue was removed from all samples prior to surface sterilization. Infected parts of the diseased grapevines were cut into disks and surface-disinfested by immersing in 1.5% solution of NaOCl for 30 seconds and rinsed in sterile distilled water. Ten pieces of wood tissue were taken from the margin between necrotic and apparently healthy tissue and plated onto malt extract agar (MEA, 2% malt extract, Mashhad, Iran; 1.5% agar, Merck, Germany) supplemented with 1 g L^{-1} streptomycin sulphate (MEAS). The Petri dishes were incubated at 25°C for 2 weeks. Hyphae growing out from the tissue pieces were subcultured onto fresh potato dextrose agar (PDA, Merck, Germany) plates, incubated at 25°C in the dark, and pure cultures were obtained through hyphal tipping.

Morphological and Cultural Characterization

Isolates of *Botryosphaeriaceae* spp. were initially identified based on colony and conidial morphology and compared with previously published reports (Pennycook and Samuels, 1985; Phillips, 2002; Taylor *et al.*, 2005). In order to enhance sporulation, cultures were placed on 2% water agar (WA, 2% agar; Merck, Germany) containing autoclaved grapevine wood chips and incubated at 25°C under 12 hours photoperiod. Isolates were examined weekly for the formation of pycnidia and conidia. Conidial morphology (shape, cell wall, color, and presence/absence of septa) from pycnidia was recorded using a compound microscope. The length and width of 50 conidia per isolate were measured. Spore dimensions are given as the mean of dimensions with extremes in parentheses. All isolates were grown on PDA plates at 25°C. Plugs of agar, 5 mm in diameter, were cut from the leading edge of 3-day-old colonies and placed in the center of PDA plates which were then incubated in the dark at temperatures ranging from 10 to 40°C in 5°C increments. There were four replicates for each isolate and temperature combination. After 24, 48, and 72 hours, colony diameter was measured along two perpendicular axes and data were converted to radial growth in millimeters per day.

Molecular Identification and Phylogenetic Analysis

For DNA extraction, isolates were grown on PDA for 10 to 15 days at 25°C in the dark. Fungal mycelium from pure cultures were scraped and mechanically disrupted by grinding to a fine powder in liquid nitrogen using a mortar and pestle. Total DNA was extracted using the DNeasy Kit (Qiagen, Germany) following the manufacturer's instructions. DNA samples were kept at -20°C until they were used for further studies.

The primers ITS4 and ITS5 (White *et al.*, 1990) were used to amplify the ITS rDNA region (including the 5.8S gene). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify a part of elongation factor (EF-1α) and β-tubulin (BT) genes, respectively. The reaction mixture for PCR contained 1 µl of DNA template, 2.5 µl of 10X buffer, 1 µl of each primer, 2.5 µl of $MgCl_2$, 1 U of *Taq* polymerase (Cinnagen, Iran) and 2 µl of dNTPs. Each reaction was made up to a final volume of 25 µl with sterile water (Chromasolv Plus, Sigma-Aldrich, Steinheim, Germany). The PCR program for the elongation factor gene included an initial denaturation for 30 seconds at 94°C followed by 40 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 45 seconds) and extension (72°C for 90 s) and ended with a final extension step at 72°C for 5 minutes (Slippers *et al.*, 2004a). The PCR program for BT and ITS genes included an initial

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denaturation for 3 minutes at 94°C followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (56°C for 45 seconds) and extension (72°C for 1 minute) and ended with a final extension step at 72°C for 15 minutes. PCR amplifications were performed on a iCycler thermal cycler (Bio-Rad, USA).

The PCR products were visualized on 1% agarose gels (UltraPure™ Agarose, Invitrogen, USA). A 100 bp ladder was used as a molecular weight marker (GeneRuler™ DNA Ladder Mix, Fermentas, Lithuania). PCR products were purified with the High Pure PCR Product Purification Kit (Bioneer, Germany) and sequenced in both directions by Macrogen Inc. Sequencing Center (Seoul, South Korea).

Reference sequences for the *Botryosphaeriaceae* spp. were obtained from GenBank and TreeBase to build representative alignments for phylogeny using Geneious Pro 3.5.6 (Biomatters Ltd. Auckland, New Zealand). Reference sequences for the ITS, EF-1α and BT regions were taken from the references of Alves *et al.* (2006) and Phillips *et al.* (2008). Sequences were aligned in Geneious software. The automated alignments were adjusted manually in Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002) and phylogenetic analyses were performed in PAUP (Phylogenetic Analysis Using Parsimony) 4.0b10 (Swofford, 2000). The heuristic search option was used set to 100 random sequence additions using tree bisection and reconstruction as the branch swopping algorithm. All characters were unordered and of equal weight and gaps in the alignment were treated as missing data. A bootstrapping method (Hillis and Bull, 1993) was used to determine whether or not trees obtained during the heuristic search could be regarded as robust or not using PAUP's bootstrap search option set to 1,000 bootstrap replications. The measured tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for each tree resulting from the heuristic search.

Pathogenicity Tests

Two isolates each of *D. seriata* (IRB2, IRB7) and *N. parvum* (IRN1 and IRN3) were used (Table 1). Pathogenicity tests were conducted on 1-year-old grapevine cuttings cv. Cabernet Sauvignon. Dormant cuttings were cut into uniform lengths containing six to seven buds. In order to enhance callusing and rooting, dormant cuttings were placed at 35°C with high humid conditions for 4 weeks. After callusing and rooting, cuttings were wounded between the two upper internodes with a 4 mm cork borer. A 4 mm mycelium agar plug from a 1-week-old culture was placed in the wound. Wounds were wrapped with moist cotton and parafilm. Twelve cuttings per fungal isolate were used. Twelve cuttings were inoculated with 4 mm non-colonized PDA agar plugs as controls. All cuttings were planted immediately in individual pots, placed in a greenhouse at 25ºC and watered as needed. Plants were arranged in a completely randomized design. Cuttings were collected after 4 months and inspected for lesion development and disease symptoms. The extent of vascular discoloration was measured upward and downward from the inoculation point. Small pieces (0.5 to 1 cm) of necrotic tissue from the edge of each lesion were cut and placed on PDA in an attempt to recover the inoculated fungi and complete Koch's postulates. Fungi were identified as previously described.

RESULTS

Field Observations, Sample Collection, and Fungal Isolation

Different symptoms including longitudinal splitting of canes, stunted shoot growth and dead cordons were observed at each of the surveyed vineyards. In this study, 15 isolates of *D. seriata* and 12 isolates of *N. parvum*

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Table 1. Colony growth rates of *Diplodia seriata* and *Neofusicoccum parvum* isolates and sequence data used in this study.

Isolates		Mean of growth (mm)[a] after 48 hours at			Temperature study		GenBank accession number[b]		
Code	Identity	20°C	25°C	30°C	Opt. (°C)[c]	Growth rate [d]	β-tubulin	ITS	EF1-α
IRB1	*Diplodia seriata*	13.5 (14.2)14.5	25.0(26.5)27.0	26.0(27.8) 28.5	30	13.9	GU121821	GU121876	GU121848
IRB2	*Diplodia seriata*	14.0(14.3)14.5	23.0(24.2)25.5	27.5(28.2)30.0	30	14.1	GU121822	GU121877	GU121849
IRB3	*Diplodia seriata*	14.0(14.8)15.0	25.0(26.6)28.0	27.0(28.6)30.0	30	14.3	GU121823	GU121878	GU121850
IRB4	*Diplodia seriata*	14.5(15.3)16.0	22.0(24.4)26.0	27.0(27.4)28.0	30	13.7	GU121824	GU121879	GU121851
IRB5	*Diplodia seriata*	13.5(14.3)15.0	25.0(27.6)29.0	27.0(28.7)30.0	30	14.6	GU121825	GU121880	GU121852
IRB6	*Diplodia seriata*	14.5(15.0)15.5	24.0(25.3)26.0	26.5(27.5)29.0	30	13.8	GU121826	GU121881	GU121853
IRB7	*Diplodia seriata*	15.0(15.6)16.0	23.5(24.5)26.0	27.5(28.4)30.0	30	14.2	GU121827	GU121882	GU121854
IRB8	*Diplodia seriata*	11.5(12.7)14.0	23.5(25.0)25.5	27.0(28.3)29.5	30	14.2	GU121828	GU121883	GU121855
IRB9	*Diplodia seriata*	13.5(14 5)15.5	23.5(25.5)26.5	26.5(27.8)29.0	30	13.9	GU121829	GU121884	GU121856
IRB10	*Diplodia seriata*	14.0(14.5)15.0	22.5(24.7)26.0	26.0(26.9)27.5	30	13.5	GU121830	GU121885	GU121857
IRB11	*Diplodia seriata*	13.5(14.5)15.0	24.5(27.2)28.5	27.0(28.3)29.5	30	14.2	GU121831	GU121886	GU121858
IRB12	*Diplodia seriata*	13.5(14.5)15.5	25.0(26.5)27.5	26.5(28.3)29.5	30	14.2	GU121832	GU121887	GU121859
IRB13	*Diplodia seriata*	13.5(14.2)15.5	22.5(25.1)27.0	26.0(27.0)27.5	30	13.5	GU121833	GU121888	GU121860
IRB14	*Diplodia seriata*	13.0(13.9)15.0	26.0(27.7)29.0	27.0(28.3)29.0	30	14.2	GU121834	GU121889	GU121861
IRB15	*Diplodia seriata*	12.0(12.8)14.0	24.0(24.8)25.5	26.5(27.1)27.5	30	13.6	GU121835	GU121890	GU121862
Mean		12.7(14.3)15.1	23.9(25.7)26.9	26.7(27.9)30.0		14			
IRN1	*Neofusicoccum parvum*	3.0(4.8)6.0	14.5(15.8)18.0	12.0(13.5)15.5	25	7. 9	GU121836	GU121891	GU121863
IRN2	*Neofusicoccum parvum*	3.0(5.5)8.5	12.5(16.2)18.0	13.0(15.8)19.0	25	8.1	GU121837	GU121892	GU121864
IRN3	*Neofusicoccum parvum*	4.0(5.9)7.0	13.0(15.8)17.0	14.0(15.5)16.0	25	7. 9	GU121838	GU121893	GU121865
IRN4	*Neofusicoccum parvum*	2.5(4.4)6.5	14.0(15.9)18.0	14.0(15.6)17.0	25	7.8	GU121839	GU121894	GU121866
IRN5	*Neofusicoccum parvum*	2.5(4.8)7.0	14.0(15.9)17.5	13.0(14.3)16.5	25	7.2	GU121840	GU121895	GU121867
IRN6	*Neofusicoccum parvum*	3.0(4.5)6.5	13.5(16.0)17.0	12.5(14.5)15.5	25	7.3	GU121841	GU121896	GU121868
IRN7	*Neofusicoccum parvum*	3.5(4.5)5.5	12.5(15.0)17.0	12.0(14.2)15.0	25	7.1	GU121842	GU121897	GU121869
IRN8	*Neofusicoccum parvum*	2.0(3.4)5.5	12.0(14.8)17.5	11.5(14.2)16.0	25	7.1	GU121843	GU121898	GU121870
IRN9	*Neofusicoccum parvum*	2.5(3.9)6.5	13.5(15.2)16.5	12.5(13.7)15.0	25	6.9	GU121844	GU121899	GU121871
IRN10	*Neofusicoccum parvum*	4.0(4.3)5.0	15.0(15.8)17.5	12.5(15.3)17.0	25	7.7	GU121845	GU121900	GU121872
IRN11	*Neofusicoccum parvum*	3.5(4.3)5.0	13.5(15.2)17.0	12.5(13.9)15.0	25	7.0	GU121846	GU121901	GU121873
IRN12	*Neofusicoccum parvum*	2.5(4.1)4.5	14.5(15.7)17.0	12.0(14.1)16.0	25	7.1	GU121847	GU121902	GU121874
Mean		3.0(4.5)6.1	13.5(15.6)17.3	12.6(14.6)16.1	----	7.4			

[a] Data shown as minimum, mean, and maximum of mycelium radial growth; [b] ITS= Internal transcribed spacer, EF1-α= Elongation factor; [c] The temperature at which the isolates produced the maximum radial growth after 48 hours, [d] Maximum mycelium radial growth (mm) after 48 hours (at the optimum temperature).

were isolated in 31.9% of positive samples (5.1% *D. seriata* and 4.1% *N. parvum*) and 19.5% (8 vineyards) of vineyards, all in the Fars province (south-western Iran). Both species were isolated from grapevines showing leaf yellowing, dieback, and reduced growth. *Diplodia seriata* and *N. parvum* were predominantly isolated from the wedge-shaped necrosis, with 6.3% and 5.0% of the total isolations (240 fungal isolates), respectively. *Diplodia seriata* was found in three of seven vineyards in Bavanat (in 2007) and was isolated from grapevines showing wedge-shaped necrosis (12 isolates) and central necrosis (3 isolates) symptoms in cross sections. *Neofusicoccum parvum* was obtained from five of nine vineyards in Kavar and Bavanat (in 2006 and 2007, respectively) and was isolated from grapevines showing wedge-shaped necrosis (9 isolates), central necrosis (2 isolates) and brown to black streaking (one isolate) symptoms in cross sections. Both species were occasionally isolated with other fungal grapevine trunk pathogens such as *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingf. and L. Mugnai and *Phaeomoniella chlamydospora* W. Gams, Crous, M. J. Wingf. and L. Mugnai from the same vine, but they were not isolated together from the same canker.

Morphological Characterization

All isolates of *Botryosphaeriaceae* produced aerial and highly dense mycelium on PDA. Cultures were initially white, but becoming grey to olivaceous-grey darkening to black. At first, isolates were separated into two groups based on their appearance in culture, conidial size, and morphology and optimum temperature for growth. Out of 27 isolates, 15 isolates obtained from Bavanat had pigmented conidia which were ovoid with a broadly rounded apex, measuring (18.0)23.5(29.4)×(7.6)9.3(10.6) µm (Length/Width ratio, L/W= 2.5). These isolates had a regular colony shape and colour (whitish-cream in 48 hours), abundant aerial mycelium and a fast growth rate [mean radial growth of 27.9 mm (SD= 0.60, SE= 0.15) at 30°C as optimum temperature in 48 hours] that turned dark-green in 10 days. Twelve isolates had hyaline and fusiform conidia, measuring (8.3)16.0(21.9)×(4.1)6.0(7.8) µm (Length/Width ratio, L/W= 2.7). These isolates had irregularly shaped colonies with dense, fluffy, aerial mycelium in the center and a moderate growth rate [mean radial growth of 15.6 mm (SD= 0.44, SE= 0.13) at 25°C as optimum temperature in 48 hours]. Based on the observed morphological characters and published data (Shoemaker, 1964; Denman *et al.,* 2000; Zhou and Stanosz, 2001; Phillips, 2002; Tylor *et al.*, 2005; Slippers *et al.*, 2005, 2007), isolates with hyaline conidia were identified as *N. parvum* and those with pigmented conidia were identified as *D. seriata*. Results of the temperature studies showed that optimal growth temperature varied for these two species. *Diplodia seriata* isolates grew much faster than those of *N. parvum*, and were able to fully colonize an 85 mm Petri dish plate within 72 hours. Furthermore, isolates of *D. seriata* showed a higher optimum temperature for growth than those of *N. parvum*. *Diplodia seriata* isolates showed maximum radial growth at 30ºC after 48 hours on PDA, while optimum temperature for *N. parvum* isolates was 25ºC. Isolates of *N. parvum* did not grow at 5 and 40ºC after 72 hours, and *D. seriata* isolates exhibited only very slight growth under these conditions. Morphological characteristics are summarized in Tables 1 and 2.

Phylogenetic Analyses

The combined ITS, EF-1α and BT dataset contained 1,506 characters including the gaps, of which 260 were parsimony-informative, 158 were variable and parsimony-uninformative, and 1,086 were constant. The phylogenetic analysis clearly showed that the isolates belonged to one of the two species (Figure 1). Fifteen isolates

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Table 2. Micro-morphological characters of *Diplodia seriata* and *Neofusicoccum parvum* isolates after 35 days of incubation at 25°C at 12 h photoperiod.

Isolates		Conidial dimension			Conidial dimension by others (µm)	
Isolate	Identity	Conidial size (µm)[a]	Mean±SD[b] (µm)	L/W ratio[c]	Conidial dimension	Source of data
IRB1	*Diplodia seriata*	20.0(22.5)28.5 ×7.5(10.0)10.0	23.7±1.7×9.4±0.8	2.5		
IRB2	*Diplodia seriata*	17.5(22.5)27.5×7.5(10.0)10.0	23.2±2.4 × 9.3±0.9	2.5	15-30×7-17	Urbez -Torres *et al*. (2006)
IRB3	*Diplodia seriata*	15.0(22.5)30.0×7.5(7.5)10.0	22.5±3.3×8.5±1.2	2.6	15-25×8-14	Savocchia *et al*. (2007)
IRB4	*Diplodia seriata*	17.5(22.5)32.5×7.5(10.0)15.0	24.8±2.9×10.2±1.3	2.4	(20.0)21.0-23.0(24.0)×8.0-10.0	Taylor *et al*. (2005)
IRB5	*Diplodia seriata*	17.5(22.5)30.0×7.5(10.0)11.5	23.3±2.7×9.5±1.9	2.5	(13.0)22.0-26.0× (9.0)10.0-13.0(15.0)	Phillips (2002)
IRB6	*Diplodia seriata*	20.0(22.5)28.8×7.5(10.0)10.0	23.8±1.8×9.7±0.7	2.5	22.0-26.0×10.0-12.0	Larignon and Dubos (2001)
IRB7	*Diplodia seriata*	15.0(22.5)30.0×7.5(7.5)10.0	22.6±3.2×8.6±1.2	2.6	20.0-26.0×9.0-12.0	Punithalingam and Waller (1973)
IRB8	*Diplodia seriata*	20.0(25.0)27.5×7.5(10.0)10.0	23.6±1.8×9.4±0.8	2.5	13.1-29.2×7.1-12.9	Urbez - Torres *et al*. (2008)
IRB9	*Diplodia seriata*	20.0(25.0)28.7×7.5(10.0)10.0	23.8±2.0×9.4±0.8	2.5		
IRB10	*Diplodia seriata*	20.0(22.5)28.8×7.5(10.0)10.0	24.0±1.9×9.3±0.8	2.6		
IRB11	*Diplodia seriata*	21.2(25.0)28.8×8.0(10.0)10.0	24.1±1.9×9.4±0.7	2.6		
IRB12	*Diplodia seriata*	18.8(25.0)30.0×8.8(10.0)11.3	24.1±1.9×9.6±0.6	2.5		
IRB13	*Diplodia seriata*	15.0(22.5)30.0×7.5(10.0)10.0	22.8±3.2×9.0±1.1	2.5		
IRB14	*Diplodia seriata*	17.5(22.5)30.0×7.5(10.0)11.3	23.4±3.0×9.4±1.3	2.5		
IRB15	*Diplodia seriata*	15.0(22.5)30.0×7.5(10.0)10.0	23.1±3.7×8.8±1.2	2.6		
Mean		18.0-29.4×7.6-10.6	23.5×9.3	2.5		
IRN1	*Neofusicoccum parvum*	10.0(15.0)22.5 × 5.0(5.0)7.5	15.2±2.9×5.5±0.9	2.8	(12)15-20(24)×(4-)4.5-6(7.5)	Phillips (2002)
IRN2	*Neofusicoccum parvum*	10.0(15.0)22.5 × 5.0(5.0)7.5	15.2±2.9×5.5±0.9	2.8	(11)14-18(-23)×5-7(10)	Pennycook and Samuels (1985)
IRN3	*Neofusicoccum parvum*	7.5(17.5)21.3 × 3.8(6.3)7.5	16.0±2.9×6.0±1.0	2.7	10-23×5-9	Urbez -Torres *et al*. (2006)
IRN4	*Neofusicoccum parvum*	7.5(17.5)21.3×3.8(6.3)7.5	15.8±3.3×6.3±1.2	2.5		
IRN5	*Neofusicoccum parvum*	10.0(15.0)22.5×5.0(5.0)8.8	15.8±3.2×6.2±1.1	2.5		
IRN6	*Neofusicoccum parvum*	7.5(17.5)21.3×3.8(6.3)7.5	16.4±3.0×6.1±1.1	2.7		
IRN7	*Neofusicoccum parvum*	7.5(17.5)21.3×3.8(7.5)7.5	16.0±3.1×6.2±1.3	2.6		
IRN8	*Neofusicoccum parvum*	7.5(17.5)21.3×3.8(6.3)7.5	16.0±3.3×6.2±1.1	2.6		
IRN9	*Neofusicoccum parvum*	7.5(17.5)22.5×3.8(6.3)8.8	16.4±3.4×6.0±1.2	2.7		
IRN10	*Neofusicoccum parvum*	7.5(20.0)21.3×3.7(7.5)7.5	17.3±3.0×6.2±1.3	2.8		
IRN11	*Neofusicoccum parvum*	8.8(17.5)22.5×3.8 (6.3)7.5	15.1±3.4×6.1±1.2	2.5		
IRN12	*Neofusicoccum parvum*	8.8(17.5)22.5×3.8(6.3)8.8	16.4±3.0×6.0±1.1	2.7		
Mean		8.3-21.9×4.1-7.8	16.0×6.0	2.7		

[a] Data shown as minimum, most frequent value (mode), and maximum size for, respectively, length and width of 60 conidia measured for each isolate; [b] Standard deviation, [c] Length/Width.

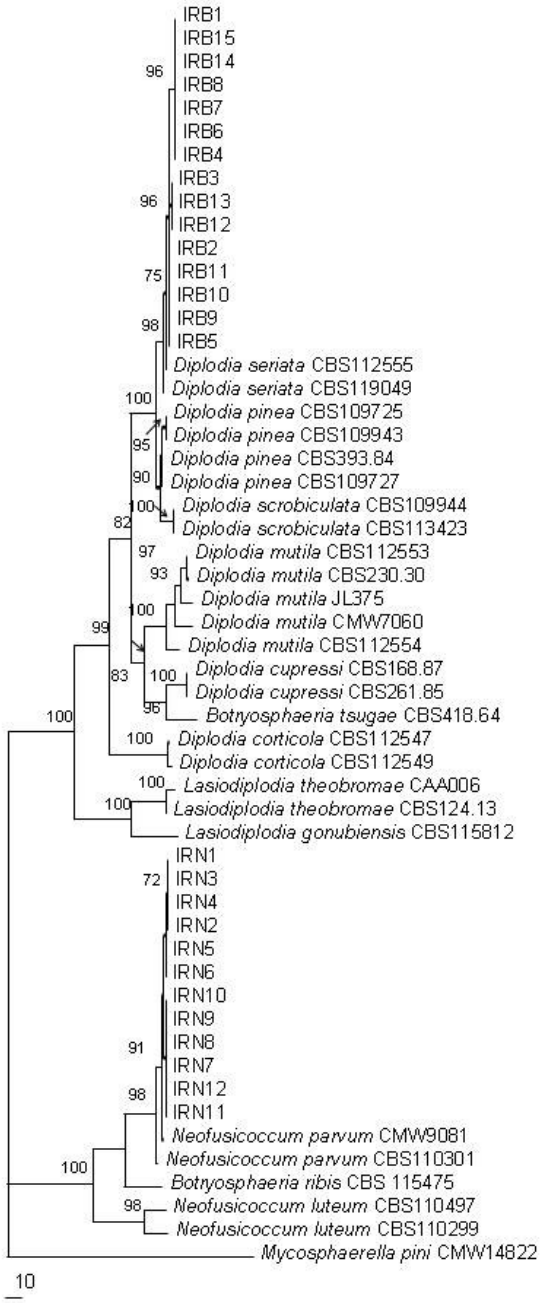

Figure 1. One of 12 most parsimonious trees obtained from heuristic searches of a combined alignment of the ITS, EF-1α and β-tubulin gene sequences (TL= 703 steps; CI= 0.768; RI= 0.922; RC= 0.708, HI= 0.232). Bootstrap support values above 65% are shown at the nodes. *Mycosphaerella pini* was used as outgroup.

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(IRB1- IRB15) were identified as *D. seriata* based on a monophyletic clade with a bootstrap support of 98% with the reference sequences. Twelve isolates (IRN1-IRN12) formed a monophyletic clade with *N. parvum* reference sequences with a bootstrap support of 98% and thus were identified as *N. parvum*.

Pathogenicity Tests

Diplodia seriata (IRB2 and IRB7) and *N. parvum* (IRN1 and IRN3) isolates were pathogenic on cuttings of cv. Cabernet Sauvignon. Symptoms appeared as petioles and leaf dieback and dried-out with extended lesions upward and downward from the point of inoculation (Figure 2). Both species produced longer lesions and were significantly different from the control ($F= 25.08$, $P< 0.0001$) and lesions lengths varied within and between species. Mean lesion lengths for *N. parvum*, *D. seriata* and the control were 24.4 (SD= 2.12, SE= 2.02), 9.0 (SD= 2.10, SE= 1.17) and 3.5 (SD= 1.13, SE= 0.32) mm, respectively. Results showed that *N. parvum* isolates were the most virulent and differed significantly from *D. seriata* isolates ($F= 43.22$, $P< 0.0001$). Although *D. seriata* isolates produced larger lesion than the control, but only one isolate (IRB2) differed significantly from the control.

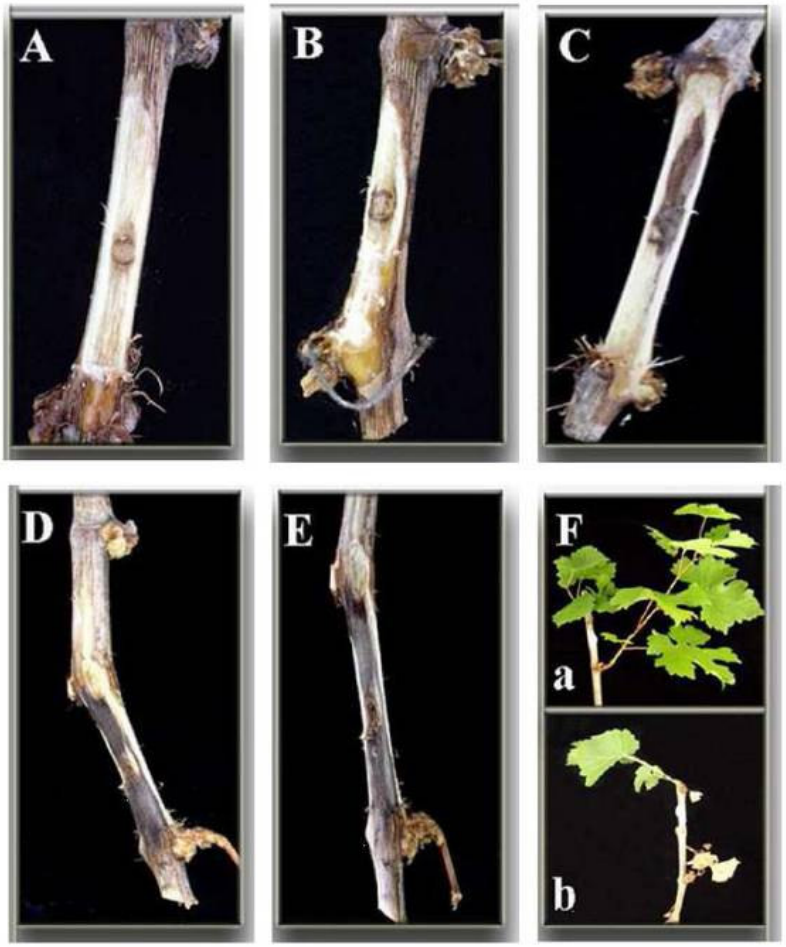

Figure 2. Lesions caused by *Diplodia seriata* and *Neofusicoccum parvum* four months after inoculation on Cabernet Sauvignon shoots. (A) Control plant; (B and C) Lesions caused by *Diplodia seriata*; (D and E) Lesions caused by *N. parvum*, (F) Leaf symptoms: (a) Control plant and (b) Plant inoculated with *N. parvum*.

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DISCUSSION

This is the first attempt to study the presence and pathogenicity of *Botryosphaeriaceae* spp. associated with black dead arm disease of grapevine in Iran. Based on morphological characteristics combined with analyses of DNA sequences, two *Botryosphaeriaceae* species, *N. parvum* and *D. seriata*, were isolated and identified from grapevines with decline symptoms in the Fars province. In this study, these species were isolated from grapevines that were more than 10 years old and showed dieback, leaf yellowing, and reduced growth. Cross sectioning of the stem of affected plants showed different wood discoloration symptoms including wedge-shaped necrosis and wood streaking. Similar symptoms were previously recorded in a vineyard survey in Fars province (Mohammadi and Banihashemi, 2007). Wedge-shaped necrosis and wood discoloration is commonly associated with the presence of *Botryosphaeriaceae* spp. on grapevine (Castillo-Pando *et al*., 2001; Phillips, 2002; Savocchia *et al*., 2007). Eutypa dieback shows similar symptoms in diseased wood, but other symptoms associated with Eutypa dieback, such as shortened internodes or yellow cupped leaves in the early stages of the growing season (Carter, 1988) were not observed in this study. The absence of Eutypa dieback may be due to climatic influences or the lack of introduction of the fungus into these regions.

Colony and conidial morphology were the most important characteristics to identify and distinguish *N. parvum* from *D. seriata*. The use of these morphological features is in agreement with the previous reports for these species (Shoemaker, 1964; Denman *et al.,* 2000; Zhou and Stanosz, 2001; Phillips, 2002; Taylor *et al.*, 2005; Slippers *et al.*, 2005, 2007). Conidial dimensions obtained in this study for *D. seriata* were generally slightly larger than those recorded by Úrbez-Torres *et al*. (2006, 2008). The range of conidial length/width ratio reported by Savocchia *et al*. (2007) for *D. seriata* (1.2 to 2.6) covered the range of isolates in this study (2.4 to 2.6) but the mean (2.1) was smaller than that of our isolates (2.5). Radial growth rates and optimum temperatures obtained in this study for *D. seriata* and *N. parvum* are generally slightly different from some previous reports (Leavitt, 1990; Pennycook and Samuels, 1985; Jacobs and Rehner, 1998; Sánchez *et al*., 2003). The estimated temperatures at which each *Botryosphaeriaceae* spp. reached the maximum radial growth on PDA were 28.2ºC for *N. parvum* and 26.8ºC for *D. seriata* after 48 h.(Úrbez-Torres *et al*., 2006). Ploetz *et al*. (2009) and van Niekerk *et al*. (2004) reported 30ºC and 27.8 ºC as optimum temperature for mycelial growth of *N. parvum,* respectively, while Espinoza *et al*. (2009) and Thomidis *et al*. (2011) reported 25 ºC as the optimum temperature for this species. The reasons for these discrepancies are unknown, but may reflect differences between the isolates studied and could be due to differences in geographical location of the isolates sources.

In this study, 15 isolates of *D. seriata* were obtained from Bavanat, a region with a cooler climate compared to other parts of the Fars Province, while two isolates of *N. parvum* were obtained from Kavar (warmer climate than in Fars Province) and ten isolates from Bavanat region. It would seem that the diversity and frequency of *Botryosphaeriaceae* spp. varied according to climatic differences among the regions studied. Some species of *Botryosphaeriaceae*, such as *L. theobromae*, are regarded as tropical and subtropical pathogens (Burgess *et al*., 2003) and the cooler climates might prevent their establishment in these regions. However, cosmopolitan species such as *D. seriata* occur in all of the regions surveyed (Taylor *et al*., 2005). In California, *N. parvum* was found mainly in the northern part of the State (Úrbez-Torres *et al*., 2006) and has recently been reported on grapevines from south-eastern Australia (Cunnington *et al*.,

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2007). Thus, it seems that this species may occur on grapevines under different climatic conditions.

Pathogenicity tests conducted on Cabernet Sauvignon cuttings showed that *N. parvum* and *D. seriata* isolates from Iran were pathogenic and *N. parvum* isolates resulted in much larger lesions than *D. seriata* isolates. *Neofusicoccum parvum* is one of the most virulent *Botryosphaeriaceae* species on grapevine worldwide (Phillips 2002; van Niekerk *et al*., 2004; Úrbez-Torres and Gubler, 2009). According to Taylor *et al*. (2005), *D. seriata* was reported as a likely saprophyte but Auger *et al*. (2004) reported the occurrence of this species affecting rooted cuttings of Red Globe in Chile. *Diplodia seriata* was also found pathogenic on cultivar Periquita in South Africa (van Niekerk *et al*., 2004). Larignon *et al*. (2001) suggested that *D. seriata* was the causal agent of dark streaks on one-year-old canes of Cabernet Sauvignon in France. Based on the pathogenicity trials in California, *L. theobromae*, *Neofusicoccum luteum*, *N. parvum*, and *N. australe* were categorized as highly virulent species on inoculated grapevines while *D. seriata*, *D. mutila*, *Dothiorella iberica* and *D. viticola* were considered weakly virulent (Úrbez-Torres and Gubler, 2009). The present study is the first report confirming the presence of *N. parvum* and *D. seriata* associated with black dead arm disease of grapevine in Iran. A more complete sampling from other provinces with different climatic conditions will be necessary to fully resolve the role of *Botryosphaeriaceae* species and their pathogenicity on grapevines in this country.

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خصوصیات *Diplodia seriata* و *Neofusicoccum parvum* همراه با زوال درختان انگور در ایران

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چکیده

در طول سالهای ۸۶-۱۳۸۳ و به منظور تعیین قارچهای بیمارگر همراه با شاخه و تنه درختان انگور در ایران، از باغات انگور در استان های فارس، همدان، اصفهان و کهگیلویه و بویر احد بازدید به عمل آمد. در این مطالعه ۲۷ جدایه قارچی متعلق به خانواده *Botryosphaeriaceae* از درختان انگور با علائم سرخشکیدگی و زوال در استان فارس بدست آمد. بر اساس خصوصیات ریخت شناختی، محیط کشت و مولکولی (تعیین توالی بخشی از ناحیه ITS، ژن β tubulin - (BT) و elongation factor1α (EF) دو گونه *Diplodia seriata* و *Neofusicoccum parvum* شناسایی گردید. بر اساس آزمون بیماریزایی هر دو گونه بر روی قلمه های ریشه دار شده رقم Cabernet Sauvignon بیماریزا بودند و ۴ ماه پس از مایه زنی، علائم بیماری به صورت سرخشکیدگی، خشک شدن برگها و ایجاد لکه بر روی شاخه های مایه زنی شده مشاهده گردید. نتایج حاصل از آزمون بیماریزایی نشان داد که جدایه های *N. parvum* نسبت به جدایه های *D. seriata* بیماریزایی بیشتری داشته و به طور معناداری لکه های بزگتری را روی قلمه های مایه زنی شده ایجاد نمودند. جدایه های مایه زنی شده از حاشیه لکه های تولید شده مجددا جداسازی و شناسایی گردید.

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